TECHNICAL NOTE

EFFECT OF DETECTOR WAVELENGTH ON THE DETERMINATION OF THE MOLECULAR WEIGHT OF HUMIC SUBSTANCES BY HIGH-PRESSURE SIZE EXCLUSION CHROMATOGRAPHY

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Abstract—High-pressure size exclusion chromatography (HPSEC) has proven to be an effective method for determining the molecular weights (MW) of humic substances (HS) from a variety of aquatic and terrestrial environments. HPSEC systems often use a variable wavelength UV-vis detector, which detects the analytes based upon their chromophoric composition. HS contain a range of moieties with chromophores having unique molar absorptivities (for a given wavelength), and the calculated MW may be dependent upon the wavelength chosen for the analysis. As a consequence, the choice of wavelength becomes an important parameter for the reliable determination of MW by HPSEC. The effect of UV-vis detector wavelength on the determination of the MW distribution of selected humic and fulvic acids by HPSEC is examined in this paper. For the HS examined, both the number (M_n) and weight average (M_w) MW increased with increasing wavelength. The relative increase in MW was most pronounced for Lake Fryxell fulvic acid, with a 63 and 21% increase in M_n and M_w, respectively, between 220 and 380 nm. The increases observed for Suwannee River humic and fulvic acids were less pronounced. M_n was more sensitive to changes in detector wavelength than M_w, and as a result the target HS appeared to be less polydisperse at higher wavelengths. Within the range of wavelengths commonly used for the determination of MW of HS by HPSEC (i.e., 220–280 nm), the magnitude of the increases in MW was not significant compared to variability in MW that results from changes to other operational parameters in HPSEC. © 2000 Elsevier Science Ltd. All rights reserved

Key words—humic, fulvic, HPSEC, molecular weight, polydispersity

INTRODUCTION

Humic substances (HS) are ubiquitous to natural waters; typically comprising 30–90% of the dissolved organic carbon (DOC) in surface waters (Thurman, 1985). HS have been shown to play key roles in the fate and transport of both organic and inorganic contaminants and can participate in the formation of harmful disinfection by-products. For example, the trihalomethane formation potential (THMFP) of natural waters is generally due to low to intermediate molecular weight (MW) HS (El-Rehaili and Weber, 1987; Krasner *et al.*, 1996). The efficiency of HS removal by coagulation and acti-vated carbon varies greatly with MW (El-Rehaili and Weber, 1987; Kilduff *et al.*, 1996). Moreover, competitive sorption of low MW HS on activated carbon can reduce the removal of organic contaminants (Newcombe *et al.*, 1997). With respect to the fate of "particle reactive" contaminants, Chin *et al.* (1997) reported a strong correlation between the binding of pyrene by aquatic HS and their MW. They hypothesized that the largest DOC fractions will presumably play the most active role with respect to the binding of hydrophobic organic contaminants, which would in-turn affect their ability to bioaccumulate and be transported. Thus, an accurate determination of the MW of HS is important for a number of water treatment processes as well as for understanding contaminant reactivity and mobility.

Traditional approaches for the determination of the MW of HS have included ultrafiltration, field-

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flow fractionation, vapor-pressure osmometry, ultracentrifugation, small-angle X-ray scattering, and gel permeation chromatography. In recent years high-pressure size exclusion chromatography (HPSEC) has been applied to determine the MW of DOC and HS from a variety of aquatic and terrestrial environments (Kilduff *et al.*, 1996; Peuravuori and Pihlaja, 1997; Chin *et al.*, 1998; Everett *et al.*, 1999; Meier *et al.*, 1999; Pelekani *et al.*, 1999; von Wandruszka *et al.*, 1999). The advantages of using HPSEC to determine the MW of HS over other analytical techniques (e.g., field flow fractionation, ultrafiltration, ultracentrifugation, etc.) include the use of small sample volumes (μl), minimal amount of pretreatment, ease and speed of analysis, availability of equipment, and the ability to determine both number-average and weight-average MW (M_n and M_w, respectively). Several detectors have been used with HPSEC including multi-angle light scattering (MALS), refractive index (RI), and in-line DOC analyzers; however, variable wavelength UV-vis detectors are among the most commonly used. Unfortunately, UV-vis detectors are somewhat selective in that they will only respond to or detect analytes that absorb at the wavelength at which they are operating. Because the structures of HS contain a range of chromophores with varying molar absorptivities (at any given wavelength), MW calculated for HPSEC chromatograms may be biased by the wavelength setting of the detector. Unlike pure substances, the overlapping absorption bands of the HS chromophores result in an increase in absorbance with decreasing wavelength. Chin and co-workers (1994) demonstrated that higher MW HS possess greater molar absorptivities (i.e., an extinction coefficient which is defined as the absorbance of a HS at a particular wavelength normalized to concentration as determined by carbon analysis) at 280 nm than lower MW HS. As a consequence, the UV-vis "transparent" fractions of the HS pool (presumably comprised of lower MW HS) may not be detected, thus affecting the MW calculated from the resulting chromatogram. In this paper we examine the effect of detector wavelength on the determination of the molecular weight distribution and polydispersities of selected humic substances using HPSEC in conjunction with a UV-vis detector. HS representing materials isolated from very different aquatic environments (an Antarctic lake and drainage from a southern deepwater swamp) were tested to ascertain the relative sensitivities of the analytes to the wavelength choice.

MATERIALS AND METHODS

The aquatic humic substances used in this study (generously provided by Dr George Aiken of the US Geological Survey at Boulder, CO) were isolated from the Suwannee River in Georgia and Lake Fryxell in Antarctica using the method of Thurman and Malcolm (1981). Suwannee River humic and fulvic acids (SRHA and SRFA) and Lake Fryxell fulvic acid (LFFA) were dissolved in buffered water identical in composition to the HPSEC mobile phase (see below). The DOC concentrations of the HS solutions ranged from 30 to 50 mg OC l^{-1} (depending upon the organic carbon content of the analyte).

Molecular weights (M_n and M_w) of the HS were determined by HPSEC. Details on the instrumentation, mobile phase composition and choice of calibration standards are described by Chin *et al.* (1994). Briefly, 20 μl samples were separated on a Waters Protein-Pak 125 aqueous size exclusion column (Waters Assoc., Milford, MA). The mobile phase consisted of 0.1 M NaCl, 0.002 M KH_2PO_4, and 0.002 M Na_2HPO_4 at pH 6.8. Analytes were detected using a Waters 486 variable wavelength UV-vis detector. The chromatograms were recorded using the Maxima GPC peak integration and molecular weight determination software. The HPSEC system was calibrated to molecular weights based on randomly coiled sodium polystyrene sulfonates with MW ranging from 1800 to 18,000 Da (Polysciences Inc.). The calibration curves were used to determine the molecular weight of each *i*th fraction of an analyte, M_i, at given eluted volume "*i*". M_n and M_w of the humic substances and colloids were determined using the following equations:

$$M_n = \sum_{i=1}^{N} h_i \Big/ \sum_{i=1}^{N} h_i/M_i \qquad (1)$$

and

$$M_w = \sum_{i=1}^{N} h_i(M_i) \Big/ \sum_{i=1}^{N} h_i \qquad (2)$$

where h_i is the height of the sample HPSEC curve eluted at volume "*i*" (Yau *et al.*, 1979).

RESULTS AND DISCUSSION

The humic and fulvic acids eluted from the HPSEC column as broad, monomodal distributions with subtle shoulders and small sub-peaks (Fig. 1). The peak heights (and areas) decreased with increasing detector wavelength; an observation which is consistent with the relationship between molar absorptivity and wavelength for these materials (Fig. 2). The inability of these HS to separate into discrete components defined by distinct MW fractions reflects the fact that they are complex mixtures of high to low molecular weight species that are difficult to resolve chromatographically. This phenomenon is compounded by the poor resolution of modern modified silica columns used in aqueous HPSEC. Indeed, the standards we used cannot be separated as a mixture using our column (Fig. 3), and retention times need to be determined for individual compounds. Thus, typical HPSEC chromatograms resemble a large unresolved peak with numerous smaller subpeaks and shoulders.

Both M_n and M_w increased with increasing wavelength for all three HS examined (Table 1). The relative increase in MW was most pronounced for LFFA, with a 63 and 21% increase in M_n and M_w, respectively, between 220 and 380 nm. The increase in M_n and M_w for SRFA and SRHA were less pronounced. The relative increase in MW among the

HS studied appears to be consistent with the origins of these materials. LFFA (extracted from a lake in Antarctica) is comprised entirely of autochthonous material (McKnight et al., 1991). It contains lower MW species with relatively low aromaticity (13% as determined by ^{13}C NMR; Chin et al., 1994). SRHA and SRFA are derived primarily from products of the decomposition of woody plant materials typically having high lignin contents and thus have structures with higher aromatic content (37 and 24%, respectively; Thorn et al., 1989). Finally, the molar absorptivity of the LFFA at 280 nm (150 l/mol-C cm) is significantly less than values reported for SRFA (389 l/mol-C cm) or SRHA (849 l/mol-C cm; Chin et al., 1997). As a consequence, LFFA presumably has more small to medium molecular weight range moieties that are invisible to the UV-vis detector and only the largest components are detected. Hence, its sensitivity to changes in both

number- and weight-average molecular weight as a function of wavelength choice is more pronounced relative to the other two HS.

The MW distribution of a mixture of polymers is defined by its polydispersity; the weight-average to number-average MW ratio (i.e., M_w/M_n). The polydispersities of SRHA, SRFA, and LFFA (Table 1) appear consistent with the observation that aquatic HS occupy a relatively narrow size fraction ($M_w/M_n = 1.0$ for polymers with a single MW) (Chin et al., 1994, 1998; Everett et al., 1999; Pelekani et al., 1999) relative to the MW distribution typically observed for HS from terrestrial environments (Kilduff et al., 1996; Conte and Piccolo, 1999b). This is not surprising given that these materials were isolated by XAD chromatography and represent a specific component of the DOC pool; as a result, the observed narrow MW distributions reflect the isolation procedures.
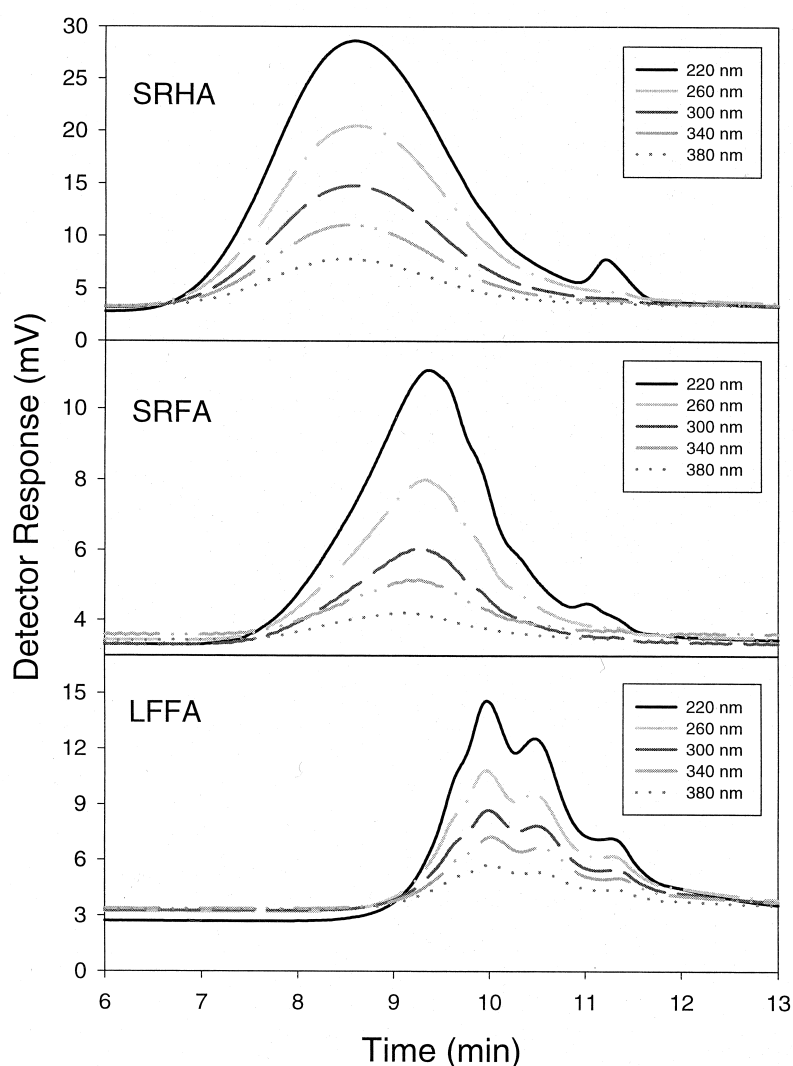

Fig. 1. HPSEC chromatograms of Suwannee River humic acid (SRHA), Suwannee River fulvic acid (SRFA), and Lake Fryxell fulvic acid (LFFA) at selected wavelengths.
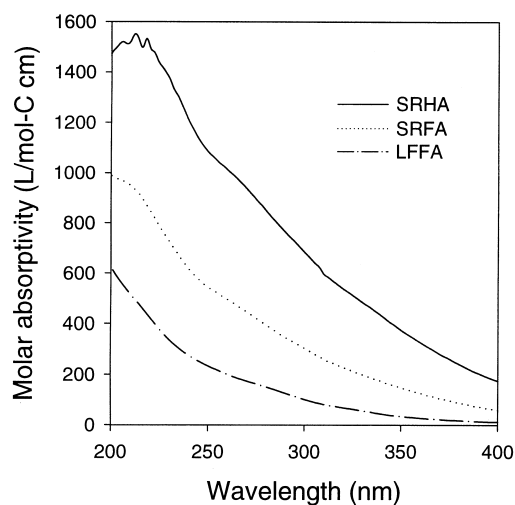
Fig. 2. Molar absorptivity as a function of wavelength for aqueous solutions of Suwannee River humic and fulvic acid (SRHA and SRFA) and Lake Fryxell fulvic acid (LFFA).

Molecular weights determined using HPSEC (with UV-vis detection) are often slightly higher than those measured by other methods that do not rely upon analyte detection by a UV-vis spectrophotometer (e.g., vapor pressure osmometry). In addition, the molar absorptivities of aquatic HS increase with increasing MW (Chin et al., 1994; Peuravuori and Pihlaja, 1997). Thus the response generated by the detector is a function of the concentration of material passing through the detector and a "number-average" extinction coefficient, which is based on the whole sample. Higher MW fractions, which have greater than the average extinction coefficients, appear to be more abundant than they actually are, while the lower MW frac-

tions (with lower extinction coefficients) appear to be lower in concentration. As the detector wavelength is increased this effect becomes more pronounced given that the lower MW components have relatively lower absorptivities at the higher wavelengths (Fig. 2). The fraction of lower MW species that in essence is "invisible" to the detector increases at longer wavelengths, resulting in a apparent increase in MW for all three HS examined (Table 1). Indeed this can be observed in the SRFA and SRHA chromatograms (Fig. 1) where a small subpeak representing a low MW fraction appears at $\lambda < 260$ nm. Though the apparent MW increases for both M_n and M_w, the magnitude of the increase is greater for M_n (which is "mathematically" biased toward lower molecular weight fractions; Yau et al., 1979) than for M_w (which emphasizes higher MW species). Thus the HS appear to be less polydisperse with increasing wavelength (Table 1), and reflects what the UV-vis detector interprets as a humic material that is both larger and residing in a very narrow range of molecular weights.

The molar absorptivity of aqueous HS solutions typically increases with decreasing wavelength (within UV-vis wavelengths) (Fig. 2). Consequently, for MW determination of HS (and DOC in general) by HPSEC, variable wavelength UV-vis detectors are typically operated at 220–280 nm to achieve maximum sensitivity (Miles and Brezonik, 1983; Chin et al., 1994; Peuravuori and Pihlaja, 1997; Meier et al., 1999; Pelekani et al., 1999; von Wandruszka et al., 1999). Within the range of 220–280 nm, the change in MW was less pronounced; the change in M_n for SRHA, SRFA, and LFFA was only 8.4, 13.8 and 2.3% respectively, while M_w changed by only 2.3, 6.5 and −2.0%. While the variability in MW within this range of wavelengths is in excess of typical standard deviations for
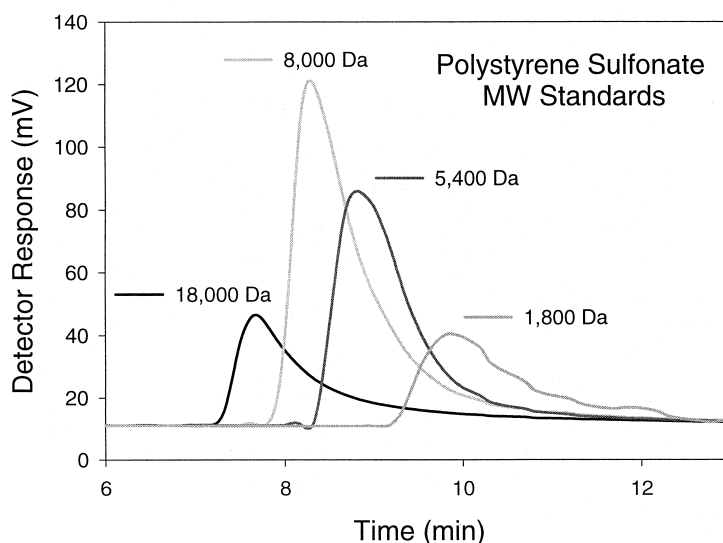

Fig. 3. HPSEC chromatograms of polystyrene sulfonate MW standards.

Table 1. Number-average (M_n) and weight-average (M_w) molecular weights, and polydispersities of aquatic HS determined at selected wavelengths

Detector wavelength (nm)	M_n	M_w	Polydispersity M_w/M_n
Suwannee River humic acid			
220	1719	3676	2.14
240	1792	3748	2.09
260	1807	3703	2.05
280	1864	3759	2.02
300	1918	3798	1.98
320	1975	3828	1.94
340	2058	3882	1.89
360	2119	3794	1.79
380	2314	3937	1.70
$\Delta_{220-380}$	34.6%	7.1%	
Suwanne River fulvic acid			
220	1451	2150	1.48
240	1540	2188	1.42
260	1614	2243	1.39
280	1652	2290	1.39
300	1719	2324	1.35
320	1761	2363	1.34
340	1796	2418	1.35
360	1875	2470	1.32
380	1957	2442	1.25
$\Delta_{220-380}$	34.9%	13.6%	
Lake Fryxell fulvic acid			
220	516	893	1.73
240	520	897	1.73
260	527	889	1.69
280	528	876	1.66
300	564	905	1.60
320	583	930	1.60
340	652	952	1.46
360	804	1043	1.30
380	839	1079	1.29
$\Delta_{220-380}$	62.6%	20.8%	

HPSEC analysis (Meier *et al.*, 1999), it is substantially less than that resulting from changes in mobile phase composition, or by the choice of HPSEC column or MW standards (Chin *et al.*, 1991; Peuravuori and Pihlaja, 1997; Conte and Piccolo, 1999a,b). Moreover, this variability is insignificant within the broader context of comparing MW of HS determined by HPSEC with MW determined using other techniques.

CONCLUSIONS

The MW of HS determined by HPSEC using a UV-vis detector increases as the wavelength at which the detector is operating is increased. This effect is more pronounced for M_n than for M_w, thus HS appear to be less polydisperse at higher wavelengths. Within the range of wavelengths commonly used for MW determination of HS by HPSEC (i.e., 220–280 nm), the magnitude of the increase in MW is not substantial enough to prevent meaningful comparisons between the MW of HS determined at different wavelengths; however, measurements made at wavelengths greater than 280 nm will result in "larger-than-expected" MW values. Moreover, the variability of MW measured using other techniques is significantly larger than the range observed from differences in the choice of detector wavelengths. Thus, in lieu of a sensitive

indiscriminate detector (such as an organic carbon analyzer), HPSEC with UV-vis detection is a useful tool for ascertaining the role of HS MW in environmental reactivity studies.

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